

# Molecular cloning and functional expression of a cDNA encoding glycosylation-inhibiting factor

(immunoregulation/macrophage migration inhibitory factor)

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**ABSTRACT** By using probes based on partial amino acid sequence of glycosylation-inhibiting factor (GIF) from a mouse T-cell hybridoma, a full-length cDNA encoding mouse GIF was isolated. A cDNA clone encoding human GIF was isolated from cDNA libraries of a GIF-producing human T-cell hybridoma by using mouse GIF cDNA as a probe. The cDNAs encode a putative 12.5-kDa peptide of 115 amino acids. Northern blot analysis demonstrated a single, 0.6-kb transcript. Polyclonal rabbit antibodies against the *Escherichia coli*-derived recombinant 13-kDa peptide bound hybridoma-derived GIF. Although the peptide did not contain a signal peptide sequence, transfection of the cDNA into COS-1 cells resulted in secretion of 13-kDa peptide, but the peptide had substantially less bioactivity than the hybridoma-derived GIF. However, expression of a chimeric cDNA encoding a fusion protein consisting of the N-terminal pro region of calcitonin precursor and human GIF and cotransfection with furin cDNA to allow intracellular cleavage of the fusion protein resulted in secretion of 13-kDa peptide that was comparable to hybridoma-derived GIF in its bioactivity. Both the 13-kDa peptide and GIF bioactivity in the transfected COS-1 supernatant bound to a monoclonal antibody against hybridoma-derived human GIF. These results indicate that the 13-kDa peptide represents recombinant GIF, but posttranslational modification of the peptide is important for generation of the bioactivity. The GIF cDNA had high homology with the cDNA encoding macrophage migration inhibitory factor. However, the recombinant GIF failed to inhibit migration of human monocytes, and recombinant human macrophage migration inhibitory factor did not have GIF bioactivity.

Previous studies on regulation of IgE antibody response in rodents described glycosylation-inhibiting factor (GIF), a lymphokine that is involved in selective formation of IgE-suppressive factor (1). GIF inhibits N-glycosylation of IgE-binding factors (IgE-BFs), and the unglycosylated IgE-BFs then selectively suppress IgE synthesis. Subsequent experiments indicated that GIF facilitated the generation of antigen-specific suppressor T cells both *in vivo* (2) and *in vitro* (3) and provided evidence that GIF is a subunit of antigen-specific suppressor T-cell factors (TsFs) (4). This hypothesis is supported by the fact that the monoclonal antibody (mAb) against lipomodulin, 141-B9, binds not only GIF but also representative TsFs from hapten-specific suppressor T-cell hybridomas (5).

We expected that biochemical characterization and molecular cloning of GIF would help to solve controversial issues regarding antigen-specific TsFs. Mouse GIF (mGIF) was purified to homogeneity from serum-free culture super-

natant of a representative GIF-producing T-cell hybridoma, 231F1, by affinity chromatography on 141-B9-coupled immunosorbent (6). Subsequently, GIF-producing human T-cell hybridomas were established, and human GIF (hGIF) from a representative hybridoma, AC5, was identified as a 14-kDa peptide by SDS/PAGE (7). Based on these findings, the present experiments were undertaken to isolate cDNA clones that encode mGIF and hGIF.<sup>§</sup>

## MATERIALS AND METHODS

**Purification of GIF.** mGIF in serum-free culture supernatant of 231F1 cells was purified by using Affi-Gel 10 (Bio-Rad) coupled to mAb 141-B9 (6) or coupled to the IgG fraction of a rabbit antiserum against recombinant mGIF. Recombinant hGIF was fractionated on Affi-Gel 10 coupled to the anti-hGIF mAb 388F<sub>1</sub> (7). Usually, 2–5 mg of a mAb or 10 mg of IgG from rabbit antiserum was coupled to 1 ml of gel. Procedures for the fractionation have been described (6, 7). After recovery of the flow-through fraction, the immunosorbent was washed with 20 column volumes of phosphate-buffered saline (0.05 M phosphate/0.15 M NaCl, pH 7.0), and proteins retained in the column were recovered by elution with 0.1 M glycine-HCl buffer (pH 3.0).

Recombinant mGIF expressed in *Escherichia coli* was purified from inclusion bodies. After disruption of *E. coli* cells, the pellet fraction was extracted with 0.2 M Tris-HCl buffer (pH 8.0) containing 6 M guanidine hydrochloride and 25 mM EDTA, and the extract was fractionated on a Sephacryl S-200 column equilibrated with the same buffer. Fractions containing the 13-kDa peptide, detected by SDS/PAGE, were concentrated and slowly added to a large volume of Tris buffer for refolding of peptides. The sample was then applied to a TSK gel DEAE-5PW column (Toyo Soda, Tokyo) equilibrated with 20 mM Tris-HCl buffer (pH 8.0), and proteins were eluted with a gradient of 0–0.1 M NaCl.

**Amino Acid Sequencing.** Affinity-purified mGIF was precipitated by 10% (wt/vol) trichloroacetic acid (6), electrophoresed in an SDS/15% polyacrylamide gel under reducing conditions, and then electroblotted to a poly(vinylidene difluoride) (PVDF) membrane. The immobilized 13-kDa protein was reduced and S-carboxymethylated *in situ* (8) and digested with 1 pM *Achromobacter* protease I (Wako Pure Chem, Tokyo) at pH 9.0. Peptides retained on PVDF membranes were subdigested with 2 pM endoproteinase Asp-N

Abbreviations: GIF, glycosylation-inhibiting factor; mGIF, mouse GIF; hGIF, human GIF; IgE-BF, IgE-binding factor; mAb, monoclonal antibody; TsF, suppressor T-cell factor; MIF, macrophage migration inhibitory factor; pro-CT, pro region of calcitonin precursor; PVDF, poly(vinylidene difluoride).

<sup>§</sup>The sequences reported in this paper have been deposited in the GenBank data base [accession nos. 10612 (hGIF) and 10613 (mGIF)].

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(Boehringer Mannheim) in 100 mM ammonium bicarbonate (pH 7.8) containing 8% acetonitrile. Peptides released from the membrane after each digestion were fractionated by reverse-phase HPLC on a  $\mu$ Bondapak C<sub>8</sub> column (particle size, 5  $\mu$ m; pore size, 300 Å; Waters) equilibrated with 0.05% trifluoroacetic acid as a mobile phase. Peptides were eluted by a linear gradient (0–50%) of 0.02% trifluoroacetic acid in 2-propanol/acetonitrile, 7:3 (vol/vol). Amino acid sequence analysis of each peptide was performed with a gas-phase sequencer (Applied Biosystems, model 470A) with modified programs for microsequencing (9).

**Construction of cDNA Library.** Total cellular RNA was isolated from 231F1 cells (4) or AC5 cells (7) by using RNazol (Tel-Test, Friendswood, TX). Poly(A)<sup>+</sup> RNA was isolated by using a FastTrack mRNA isolation kit (Invitrogen). cDNA libraries were constructed with a Uni-Zap cDNA synthesis kit (Stratagene). After screening of the cDNA library, selected cDNA clones were sequenced by the standard dideoxy method with the Sequenase kit (United States Biochemical). The DNA sequences were analyzed with MACVECTOR software (International Biotechnologies).

**Expression of Recombinant GIF.** For bacterial expression of mGIF, *Afl* II and *Bam*HI adaptor sites were ligated at both ends of mGIF cDNA by polymerase chain reaction (PCR), and the cDNA fragment was inserted by ligation into pST811 vector (10) carrying the *trp* promoter and *trpA* terminator. The plasmid was transformed into competent *E. coli* RR1 cells, and the cells carrying plasmid were cultured in M9 broth with glucose (0.8%), amino acids (0.4%), thiamin (10  $\mu$ g/ml), and ampicillin (50  $\mu$ g/ml). Cells were harvested 5 hr after the addition of indoleacrylic acid (11).

For the expression of GIF cDNA in COS-1 cells, two different types of plasmids were constructed. In one type, mGIF or hGIF cDNA was ligated into *Bgl* II/*Kpn* I-digested modified SR $\alpha$  vector (12). Since GIF does not appear to have a signal peptide sequence, we constructed another expression system for translation of a fusion protein which consisted of the N-terminal pro region of the human calcitonin precursor (pro-CT) and human GIF. Intracellular cleavage of the fusion protein was mediated by an endoprotease, furin (13), allowing the secretion of mature GIF peptide (14). The cDNA fragment encoding pro-CT was amplified by PCR using a human calcitonin cDNA as the template and oligonucleotide primers tailed with a *Pst* I recognition site (14). The amplified gene was cloned into the SR $\alpha$  vector at the *Pst* I site. To fuse hGIF cDNA to the 3' end of pro-CT cDNA in frame, the 5' extension method was applied to hGIF cDNA. The sequence of the 5' primer was 5'-CCAGATCTAAGCG-GATGCCGATGTTTCATCGTAAACACC-3', which contains a *Bgl* II site (see Fig. 1B). The amplified hGIF gene was ligated into *Bgl* II/*Kpn* I-digested SR $\alpha$  vector in which pro-CT cDNA had been inserted. Human furin cDNA was cloned into the SR $\alpha$  vector as described (14). The plasmids were transfected into COS-1 cells either by the DEAE-dextran method or by electroporation. After transfection, cells were incubated overnight in a 1:3 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F12 (DMEM/F12) containing 10% fetal bovine serum and then were cultured for 1 week in serum-free DMEM/F12 containing bovine insulin (20  $\mu$ g/ml; Sigma), human transferrin (20  $\mu$ g/ml; Sigma), 40  $\mu$ M monoethanolamine, 0.1  $\mu$ M sodium selenite, and bovine serum albumin (1 mg/ml; Sigma) to recover culture supernatant.

**Electrophoresis and Immunoblotting.** Affinity-purified GIF preparations were analyzed by SDS/PAGE in a 15% polyacrylamide slab gel under reducing conditions (15), and proteins in the gel were visualized by silver staining (16). An aliquot of a sample was analyzed along with serial 2-fold dilutions of *E. coli*-derived recombinant mGIF of known concentrations, and the concentration of the 13-kDa peptide

in the sample was estimated by the intensity of the band in silver staining. Immunoblotting was carried out with the enhanced chemiluminescence (ECL) Western blot detection system (Amersham). Polyclonal rabbit antibodies against recombinant mGIF were affinity-purified by absorption of the IgG fraction of the anti-GIF antiserum with Affi-Gel 10 coupled to *E. coli*-derived mGIF, and proteins retained in the column were eluted with glycine-HCl buffer (pH 3.0). Two to 4  $\mu$ g/ml of the affinity-purified antibodies was used to detect the GIF band.

**Detection of GIF Bioactivity.** GIF was detected by its ability to switch the mouse T-cell hybridoma 12H5 cells from the formation of glycosylated IgE-BF to the formation of unglycosylated IgE-BF. Detailed procedures for the assay have been described (3). Briefly, the 12H5 cells were cultured with mouse IgE (10  $\mu$ g/ml) in the presence or absence of a test sample, and IgE-BF in culture filtrates was fractionated on lentil lectin-Sepharose. When the 12H5 cells were cultured with IgE alone, essentially all IgE-BF formed by the cells bound to lentil lectin-Sepharose and was recovered by elution with methyl  $\alpha$ -D-mannoside. When a sufficient amount of GIF was added to the 12H5 cells together with IgE, a majority of the IgE-BF formed by the cells was not retained in the column and was recovered in the effluent fraction (3).

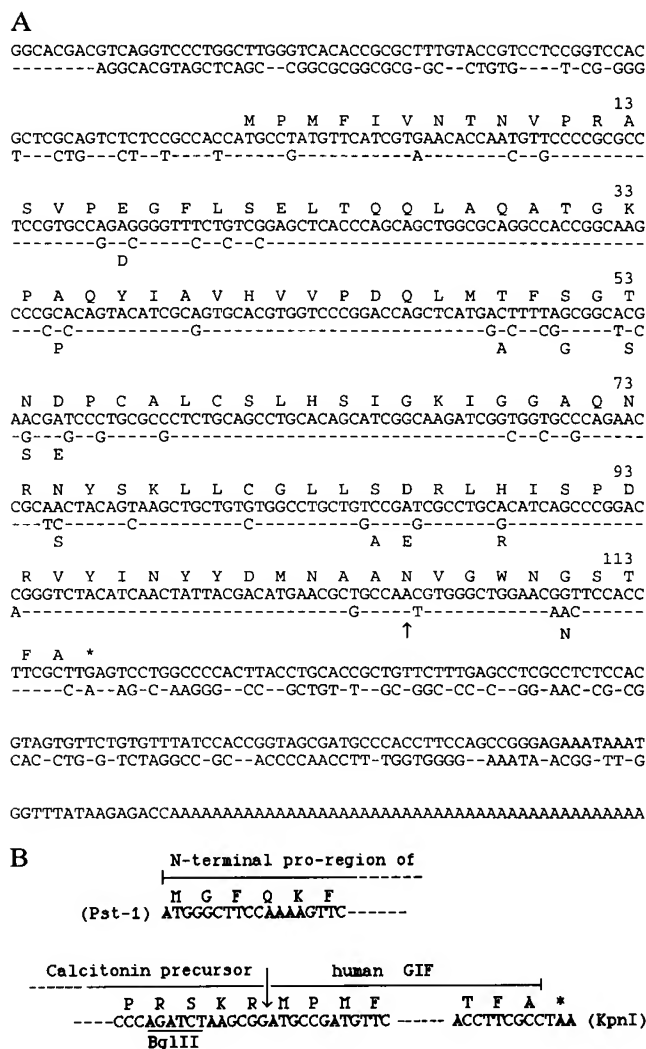
**Assay for Macrophage Migration Inhibitory Factor (MIF).** Human peripheral blood monocytes were employed as indicator cells in an agarose-droplet assay system (17). The assay was set up in triplicate or quadruplicate together with serial dilutions of a supernatant of COS-1 cells transfected with MIF cDNA (18) as a positive control. The area of migration was calculated by the following formula: migration = (diameter of total area/diameter of agarose droplet)<sup>2</sup> - 1. Percent inhibition = 100 - [(average migration of test sample/average migration of negative control)  $\times$  100]. In this assay, inhibition of  $\geq$ 20% was considered to be significant (19).

## RESULTS

**cDNA Cloning of GIF.** mGIF was isolated from culture supernatant of 231F1 cells, and the 13-kDa peptide immobilized on PVDF membrane was employed for determination of partial amino acid sequence. In this experiment, we obtained six different peptides consisting of 12–15 amino acids. Based on the N-terminal amino acid sequence (MPMFIVNTNV-PRASV) and the sequence of one of the fragments (DPCAL-CSLHSIGK), oligonucleotides were synthesized, and PCR was carried out using the two oligonucleotides as primers and single-stranded cDNA of 231F1 cells as the template. A 0.2-kb fragment amplified in the PCR was ligated to pCR1000 vector for subsequent cloning and DNA sequencing. After the nucleotide sequence was confirmed, the 0.20-kb fragment was used to screen a cDNA library from 231F1 cells.

Seven cDNA clones were isolated after screening of  $0.5 \times 10^6$  independent clones. Since restriction mapping of all of the cDNA clones showed a single pattern, the longest clone, with an insert of 0.6 kb, was chosen for DNA sequencing. The nucleotide sequence and deduced amino acid sequence of mGIF are shown in Fig. 1A. The largest open reading frame encodes 115 amino acids and the predicted amino acid sequence contained all six peptides obtained by Edman degradation of purified mGIF. The calculated size of the GIF protein is 12.5 kDa, which is in good agreement with that of purified mGIF (6). The nucleotide sequence flanking the first methionine codon favors the translation initiation rule (21). The amino acid sequence downstream from this methionine has a perfect match to that of the N-terminal sequence of the purified GIF, suggesting that GIF has no signal peptide sequence.

Since high homology was expected between mGIF and hGIF, a cDNA library constructed from mRNA of a human



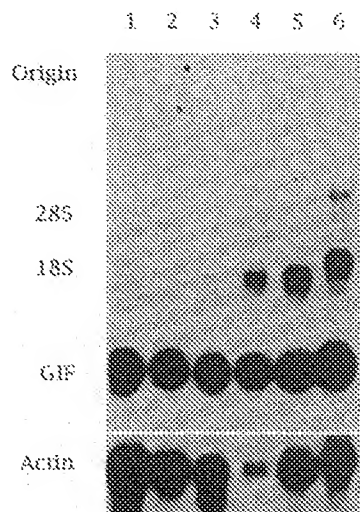
**FIG. 1.** (A) Structure of GIF cDNA clones. The second line shows the full-length nucleotide sequence of a mGIF cDNA clone, and the first line shows the predicted amino acid sequence of mGIF. The third and fourth lines show the nucleotide and amino acid sequences, respectively, of a human cDNA clone. Only differences from the mGIF sequence are shown. Arrow indicates the only difference between hGIF cDNA and human MIF cDNA, which has AGT (serine). Six peptides obtained from 231F1-derived 13-kDa GIF corresponded to amino acids 1–15, 13–26, 55–67, 67–78, 86–100, and 101–115 in the deduced amino acid sequence of mGIF. (B) Nucleotide sequence of the insert encoding a fusion of pro-CT with hGIF. Deduced amino acid sequence is shown above the nucleotide sequence. The recognition motif for furin is Arg-Xaa-Lys-Arg (20). The cleavage site is shown by an arrow. The insert was ligated into SR $\alpha$  vector through *Pst* I and *Kpn* I sites, as indicated in parentheses.

GIF-producing hybridoma, AC5, was screened with the mGIF cDNA as a probe. Among 27 clones hybridized, 4 clones having a 0.5-kb insert were sequenced, and the structure was compared with that of mGIF (Fig. 1A). The hGIF and mGIF sequences were 80% identical at the whole cDNA level, 89% identical in the putative coding region, and 90% identical at the amino acid level. The sequence of the coding region of hGIF cDNA was almost identical to the sequence of human MIF cDNA (18). The only difference is that amino acid 106 of MIF is serine, whereas the corresponding residue of hGIF is asparagine (see Fig. 1A).

The expression and the size of transcripts that hybridized to the GIF cDNA were examined by Northern analysis. Surprisingly, GIF mRNA was detected in all of the mouse cell line cells tested—231F1, CTLL-2, BW5147, A20.3, and NIH

3T3 (fibroblast). Various human cell line cells such as AC5, CEM, RPMI8866, WI-38 (embryonic fibroblast), and PC3 (prostate carcinoma cells) also contained mRNA which hybridized to the hGIF cDNA. Only a single transcript of 0.6 kb was observed in mouse or human cell line (Fig. 2). Northern blotting of RNAs from mouse tissues showed a dominant expression of GIF mRNA in brain, liver, and kidney. Since the size of the transcript is close to the size of the GIF cDNA (584 bp), it is likely that the mGIF and hGIF clones isolated represent full-length cDNAs of GIF.

**Isolation of Hybridoma-Derived GIF by Use of Antibodies Against Recombinant 13-kDa Peptide.** If the cDNA clones actually encode GIF, one may expect that antibodies against recombinant 13-kDa peptide will bind GIF from T-cell hybridomas. To test this possibility, rabbit antibodies against the *E. coli*-derived 13-kDa peptide were obtained. The purity of the recombinant mouse peptide employed for immunization was >95% as determined by SDS/PAGE (Fig. 3A), and the N-terminal amino acid sequence of the peptide corresponded to that predicted from the nucleotide sequence of the cDNA. Rabbits were immunized by an intramuscular injection of 100  $\mu$ g of peptide included in complete Freund's adjuvant, and the antiserum was obtained after five booster injections. Since IgG at 2–4  $\mu$ g/ml in the antiserum was adequate for detection of the recombinant 13-kDa peptide by Western blotting, culture filtrate of the 231F1 cells was fractionated on Affi-Gel 10 coupled with the IgG fraction of the antiserum. GIF activity was not detectable in the flow-through fraction, and >80% of the bioactivity in the culture filtrate was recovered in the eluate fraction, which gave a 13-kDa band upon SDS/PAGE (Fig. 3A). Estimation of the concentration of the 13-kDa peptide in the eluate fraction indicated that a peptide concentration of 5 ng/ml was sufficient for detection of GIF bioactivity (Table 1). Similar experiments were carried out with culture supernatants of a GIF-producing human T-cell hybridoma. Essentially all GIF activity in the supernatant bound to the anti-GIF Affi-Gel and was recovered by acid elution. The minimum concentration



**FIG. 2.** Expression of the 0.6-kb GIF transcript. Lanes: 1, 231F1; 2, BW5147 (thymoma); 3, CTLL-2 (cytotoxic T-cell line); 4, A20.3 (B-cell line); 5, PT-18 (mouse mast cell line); 6, NIH 3T3 (fibroblast line). Samples (10  $\mu$ g) of cellular RNA were electrophoresed in formaldehyde/3% agarose gel and blotted to a charged nylon membrane. After probing with the  $^{32}$ P-labeled mGIF cDNA, the same membrane was stripped and hybridized with a PCR-amplified  $\beta$ -actin cDNA labeled with  $^{32}$ P under the same conditions. Conditions for hybridization were 50% formaldehyde/5 $\times$  standard saline citrate (SSC)/1 $\times$  Denhardt's solution/0.5% SDS at 42°C, followed by subsequent washing with 2 $\times$  SSC/0.1% SDS at 25°C and 0.5 $\times$  SSC/0.1% SDS at 65°C. Positions of 28S and 18S rRNA are shown.

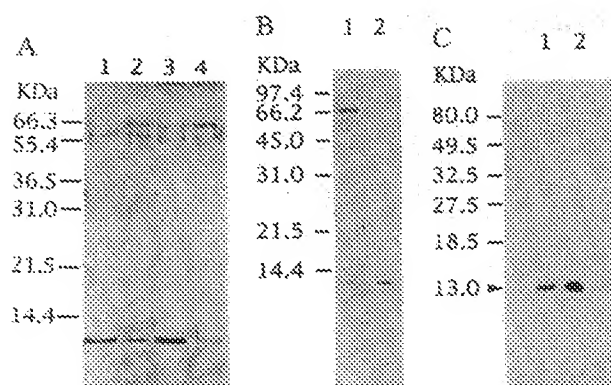


FIG. 3. Identification of hybridoma-derived GIF and recombinant GIF by SDS/PAGE. (A) Comparisons among *E. coli*-derived mGIF, 231F1-derived mGIF, and GIF from COS-1 cells transfected with mGIF cDNA. *E. coli*-derived mGIF, isolated from inclusion bodies, was applied to lane 1. The 231F1-derived GIF (lane 2) and COS-1-derived GIF (lane 3) were purified by using Affi-Gel 10 coupled to polyclonal antibodies against recombinant mGIF. Supernatant of COS-1 cells transfected with SR $\alpha$  vector alone was absorbed with the same immunosorbent, and the acid eluate fraction was applied to lane 4. Peptides were detected by silver staining. (B and C) Recombinant hGIF expressed in COS-1 cells. Mature 13-kDa GIF was detected by silver staining (B) and by Western blotting (C). hGIF cDNA ligated into SR $\alpha$  vector was transfected into COS-1 cells, and recombinant GIF in COS-1 supernatant was purified on 388F<sub>1</sub>-Affi-Gel (lane 1). hGIF was expressed by cotransfection of a chimeric cDNA encoding pro-CT-hGIF fusion protein and furin cDNA and was purified by the same procedure (lane 2).

of the 13-kDa peptide required for the detection of GIF activity in the eluate fraction was estimated to be 10 ng/ml (Table 1).

**Production of Bioactive Recombinant GIF in COS-1 Monkey Cells.** The mGIF cDNA was ligated into a modified SR $\alpha$  vector, and the plasmid was transfected into COS-1 cells. Culture supernatant of the transfected cells contained GIF bioactivity and the 13-kDa peptide, which was detected by Western blotting using polyclonal anti-GIF antibodies. Supernatants from GIF-transfected COS-1 cells and from mock-transfected cells were fractionated with the anti-GIF coupled to Affi-Gel. Essentially all GIF bioactivity in the supernatant of GIF-transfected cells bound to the immunosorbent and was recovered by acid elution, whereas the activity was not detectable in the acid eluate fraction of the supernatant from

mock transfectants. The 13-kDa peptide was detected in the eluate of GIF-transfected COS supernatant but barely detectable in the fraction from mock-transfected cells (Fig. 3A). The hGIF cDNA was expressed in COS-1 cells using the same vector, and the supernatants were fractionated on 388F<sub>1</sub>-Affi-Gel. As expected, all GIF bioactivity in the culture supernatant of GIF-transfected cells bound to the immunosorbent and was recovered by acid elution. The acid eluate fraction gave a 13-kDa band upon SDS/PAGE (Fig. 3B), and polyclonal anti-GIF antibodies bound to the band on a Western blot (Fig. 3C). These results collectively indicate that the 13-kDa peptide formed by transfected cells has GIF bioactivity. However, titration of GIF bioactivity in the affinity-purified recombinant mGIF and hGIF and estimation of the concentration of 13-kDa peptide in the preparations by SDS/PAGE indicated that the concentration of recombinant GIF required for the detection of GIF activity was 150–250 ng/ml (Table 1).

Quantitative difference in the biologic activities between the hybridoma-derived GIF and recombinant GIF suggested to us the possibility that bioactivity of the 13-kDa peptide may depend on posttranslational modification of the peptide. Since GIF does not have a signal peptide (Fig. 1A), we applied a device for the secretion of a recombinant truncated peptide via the constitutive pathway (14). Our approach was to fuse the cDNA fragment encoding human pro-CT with GIF-cDNA for the expression of a fusion protein in COS-1 cells and to utilize furin for intracellular cleavage of the fusion protein and subsequent secretion of the mature GIF. The nucleotide sequence of the insert encoding the fusion protein and the predicted amino acid sequence around the cleavage site are shown in Fig. 1B. Indeed, cotransfection of the cDNA encoding the fusion protein and furin cDNA resulted in secretion of the 13-kDa GIF. Bioactivity of the supernatant was 5–10 times higher than that of COS-1 cells transfected with hGIF cDNA. The supernatant contained the 13-kDa peptide, which could be affinity-purified by 388F<sub>1</sub>-Affi-Gel, and the peptide band in SDS/PAGE bound polyclonal anti-GIF on a Western blot (Fig. 3B and C). As expected, essentially all GIF bioactivity in the culture supernatant was recovered in the acid eluate fraction from 388F<sub>1</sub>-Affi-Gel. Further fractionation of the eluate on polyclonal anti-GIF coupled Affi-Gel indicated that both the 13-kDa peptide and the GIF bioactivity in the fraction bound to the antibodies and were recovered by acid elution. It was also found that GIF activity in the original culture supernatant bound to anti-lipomodulin (141-B9)-coupled Affi-Gel. Titration of GIF bioactivity in the affinity-purified GIF preparations showed that the specific bioactivity of recombinant hGIF obtained by this method was comparable to that of hybridoma-derived GIF (Table 1).

Since the GIF cDNA has high homology to MIF cDNA (18), we determined MIF activity of recombinant GIF. Culture supernatant of COS-1 cells cotransfected with pro-CT-hGIF cDNA and furin cDNA was fractionated on 388F<sub>1</sub>-Affi-Gel, and both the effluent and acid eluate fractions were assessed for MIF activity and GIF activity. Neither the effluent nor eluate fraction had MIF activity, although GIF bioactivity was detected in a 1:100 dilution of the eluate fraction. In the same assay, supernatant of COS-1 cells transfected with MIF cDNA showed MIF activity at the final dilution of 1:10, but no GIF activity was detected in a 1:4 dilution of the supernatant.

## DISCUSSION

In this paper, we describe the molecular cloning of cDNAs coding for mGIF and hGIF. Both cDNA clones contain a single open reading frame of 345 nucleotides which encodes a peptide of 115 amino acids. The predicted amino acid

Table 1. Bioactivity of hybridoma-derived GIF and recombinant (r) GIF

Purified GIF	Cell source	Antibody used for purification	13-kDa peptide for GIF activity,* ng/ml
mGIF	231F1	Anti-GIF	5
hGIF	31E9	Anti-GIF	10
rmGIF	COS-1	Anti-GIF	250
rhGIF	COS-1	388F <sub>1</sub>	150
rhGIF†	COS-1	388F <sub>1</sub>	10
		Anti-GIF	5
		141-B9	10

GIF in culture supernatant was purified by using Affi-Gel immunosorbent coupled either to polyclonal anti-recombinant mGIF (anti-GIF) or with mAb against hybridoma-derived GIF (388F<sub>1</sub>), or monoclonal anti-lipomodulin (141-B9).

\*Minimum concentration of the 13-kDa peptide required for the detection of GIF bioactivity. These values were calculated from the concentration of the 13-kDa peptide in an affinity-purified GIF and GIF bioactivity in serial 2-fold dilutions of the purified preparations.

†Recombinant hGIF obtained by cotransfection of a chimeric cDNA encoding a pro-CT-hGIF fusion protein and furin cDNA.

sequence of mGIF was exactly the same as that of a growth factor-induced delayed early response gene (22), and the nucleotide sequence of hGIF cDNA was identical to that of MIF cDNA (18), except for one base (Fig. 1A). The hydrophobicity plot of the amino acid sequence of both mGIF and hGIF revealed that hydrophobic and hydrophilic regions are clearly separated and that the length of each region is 20–25 residues. This finding suggests that GIF is a globular protein and that three-dimensional structure of the molecules may be important for their biologic function.

Important findings from the biologic viewpoint were (i) that transfection of the GIF cDNA into COS-1 cells resulted in the secretion of bioactive 13-kDa peptide and (ii) that the recombinant GIF bound to both the mAb against hybridoma-derived GIF and anti-lipomodulin, while the polyclonal antibodies against recombinant mouse 13-kDa peptide specifically bound hybridoma-derived mGIF and hGIF. When one considers the 90% identity in amino acid sequence between mGIF and hGIF, crossreaction of the antibodies with hGIF is reasonable. Furthermore, bioactivity of the recombinant 13-kDa peptide which was obtained by cotransfection of the chimeric gene encoding a pro-CT-hGIF fusion protein and furin cDNA was comparable to that of hybridoma-derived GIF. These findings collectively indicate that the recombinant 13-kDa peptide actually represents GIF. However, the 13-kDa peptide obtained by transfection of either mGIF cDNA or hGIF cDNA alone was 10- to 30-fold less active than the hybridoma-derived GIF. Since GIF does not have a signal peptide, one may predict that the recombinant 13-kDa peptide synthesized in this system will not go through the endoplasmic reticulum. Mechanisms underlying the secretion of soluble factors without signal peptides—interleukins 1 $\alpha$  and 1 $\beta$ —remain unclear (23). Nevertheless, the pro-CT-GIF fusion protein synthesized in COS-1 cells will go through the endoplasmic reticulum and Golgi apparatus, where the fusion protein is cleaved by the furin coexpressed in these cells (13). One may speculate that posttranslational modification of the 13-kDa peptide—e.g., proper folding of the peptide, intrachain disulfide formation, or phosphorylation—is important for the generation of GIF bioactivity. This idea may explain the fact that essentially all cell line cells and mouse tissues contained mRNA for GIF (Fig. 2), whereas the major cell source of bioactive GIF is limited to certain subsets of lymphocytes (1). It has been shown that Lyt-2<sup>+</sup> splenic T lymphocytes and antigen-specific murine suppressor T hybridomas secreted bioactive GIF but that helper T-cell clones and hybridomas did not. Our recent experiments showed that the murine CD4<sup>+</sup> T-cell hybridoma 12H5 and human CD4<sup>+</sup> T-cell line CEM secreted the 13-kDa peptide which reacted with polyclonal anti-GIF; however, even at 0.2–1.0  $\mu$ g/ml, the peptide from these cells did not exert GIF bioactivity. One might speculate that the 13-kDa peptide translated in suppressor T cells is modified for the secretion of bioactive GIF, while similar posttranslational modification of the peptide does not occur in helper T cells. Elucidation of the mechanisms for the formation and secretion of bioactive 13-kDa peptide by suppressor T cells requires further studies.

High amino acid sequence homology between hGIF and MIF suggested that GIF might have MIF activity. However, our experiments showed that affinity-purified recombinant hGIF failed to inhibit migration of human monocytes even at a 20-fold higher concentration than that required for the detection of GIF activity. It was also found that affinity-purified mGIF from 231F1 cells, with a GIF titer of 1:50, failed to inhibit the migration of mouse macrophages (results not shown). In contrast, the supernatant of COS-1 cells

transfected with MIF cDNA showed a high MIF activity but did not have GIF activity. Our more recent experiments indicated that MIF activity in the supernatant failed to be retained in either 388F<sub>1</sub>-coupled Affi-Gel or polyclonal anti-GIF-coupled Affi-Gel. The results collectively indicate that GIF is distinct from MIF. Since recombinant MIF has not been affinity-purified, it is not conclusive that the 13-kDa peptide of the predicted amino acid sequence has the MIF activity. At present, however, the possibility cannot be excluded that a single amino acid difference between GIF and MIF may account for their biologic activities. Lanakan *et al.* (22) reported that the cDNA probe of a growth factor-induced delayed early response gene, which has exactly the same sequence as our mGIF cDNA, hybridized with a large number of murine and human genomic restriction fragments, suggesting that there is a family of MIF-like genes. The GIF gene appears to belong to this family but is distinct from the MIF gene.

This paper is publication no. 79 from the La Jolla Institute for Allergy and Immunology. This work was supported by Research Grants AI11202 and AI14784 from the U.S. Department of Health and Human Services.

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